Scholars Journal of Applied Medical Sciences (SJAMS) **ISSN 2320-6691 (Online)**
Sch. J. App. Med. Sci., 2015; 3(9D):3356-3363 **ISSN 2347-954X (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublisher.com

Research Article

Effect of *Allium stracheyi* on behavior of zebrafish: a pharmacological approach

Sanjay Kumar[1], Himanshu Joshi[1], Jeevan Chandra[1], Pankaj Bahuguna[1], Vivek Kumar Kedia[2], Rakesh Kumar[3]

[1]Department of Zoology, LSM Govt. Post Graduate College, Pithoragarh, Uttarakhand, India
[2]Department of Botany, Govt. Post Graduate College, Talwari, Chamoli, Uttarakhand, India
[3]Department of Zoology, Pt. LMS, Govt. Post Graduate (Autonomous) College, Rishikesh, Dehradun, Uttarakhand, India

***Corresponding author**
Dr. Rakesh Kumar
Email: rkneuron@gmail.com

Abstract: Anxiety disorders are prevalent condition that affect emotion and cognitive behaviour and are associated with substantial impairments in both productive and social roles. Pharmacological intervention is the main therapeutic approach. Benzodiazepines are among most frequently prescribed anxiolytics. Several clinical problems are associated with the existing anxiolytics being prescribed and therefore herbal medicines are being considered as an alternative to the complementary medicine. In the present study methanolic extract of *Allium stracheyi* (Baker) was studied for its anxiolytic property in widely accepted zebrafish behavioural models of anxiety. The observations indicate that *Allium stracheyi* imparts mild anxiolytic activity.
Keywords: Anxiety, behaviour, *Allium stracheyi*, diazepam, zebrafish

INTRODUCTION

Behaviour is the most complex function of the nervous system. Adverse psychosocial factors, diseases, drugs, stress and environmental changes affect the neuronal system of an organism which leads to behavioural disturbances. Neurotransmitters play an important role in behaviour regulation. Any alteration or impairment in the neurotransmitter life cycle such as hypo/hyperactivity may change the behavioural responses leading to psychiatric illness [1-5]. Anxiety is an important component of psychiatric or medical condition [6] which ultimately decreases the quality of life. They are considered among the most prevalent psychiatric syndromes, affecting 10% to 30% of the general population of industrialized societies [7,8]. They affect emotion and cognition and exhibit > 50% co-morbidity with depression. Since anxiety and panic disorders are the common psychiatric disorders, one of the therapeutic approaches used is pharmacological intervention to solve these problems. Selectively acting drugs can be used to treat anxiety and other psychiatric disorders but the major disadvantage of the synthetic drugs is physical dependence or other side effects [9]. Previously alcohol, barbiturates and meprobamate were employed to alleviate anxiety. Anxiolytics and cognitive behavioural therapy have been in practice [10] but, many patients remain untreated, experience adverse effects of drugs [11], or do not get benefited [12]. Benzodiazepines are among the most frequently prescribed anxiolytics and thus produce their effect by facilitating GABA neurotransmission within CNS [13]. Breier and Paul [14], suggested that benzodiazepines/GABA receptor complex is involved in the pathogenesis of anxiety and benzodiazepines are considered as drug of choice in the treatment of anxiety [15]. But now it is widely accepted that clinical problems associated with benzodiazepines are the fairly high risk of dependence, tolerance and addiction in long term use [16]. Abuse liability has also been documented among vulnerable groups [17]. Other side effects include adverse effects on behavior, cognition, immunity and occurrence of peptic ulcer or hypertension [18]. Furthermore, these drugs may have adverse effects on the fetus during pregnancy and on the neonate during lactation [19].

A number of herbal medicines are commonly used for the treatment of neurological and psychiatric disorders [20]. It has been estimated that 43% of anxiety sufferers use some form of complementary therapy [21].The most popular treatments include herbal medicines [22] and anxiety disorders are amongst the most common reason for people to try herbal medicines [23].

Considerable interest has been generated by the possibility of identifying new drugs from plant source by using zebrafish *(Danio rerio)* as a suitable

animal model [24, 25]. Zebrafish model of anxiety and behavioral assays are currently used for high-throughput phenotyping and testing of various psychotropic drugs [26, 27, 28, 29]. Commonly used anxiolytics such as buspirone and diazepam are effective in zebrafish [30]. The reported behavioral responses to known anxiolytic or anxiogenic drugs indicates that zebrafish demonstrate high translation value in stress and anxiety-related pharmacological research.

Allium stracheyi (Baker) is a readily available perennial herb mainly found at the height of 2500-3000 meters of Alpine Himalayas of Uttarakhand, India [31]. Whole plant (flowers, leaves, root and bulb) is being used in traditional medicine by the local people for the treatment of various ailments including alleviation of inflammation and painful conditions [32], in the treatment of Jaundice, cold, cough, wound healing and other stomach problem [33, 34]. Nair and Nair, (1999) [35] reported various phytochemical constituents found in the plant material. Experimental studies indicate the use of *Allium stracheyi* as anti-inflammatory and analgesic activity, evaluated in carrageen induced rat paw edema method [36]. Anti-inflammatory, analgesic potential and relative toxicity of the plant extract has also been studied [37]. Since very few studies have been done, therefore, this plant was considered for pharmacological evaluation to find anxiolytic activity in zebrafish model of anxiety.

MATERIAL AND METHOD

Animals

Adult Zebrfish *(Danio rerio)* 3-5 cm in length were collected from Ben River, Pantnagar, Uttarakhand. The animals were maintained in the department animal unit in groups of 20-25, in 10-15 litre aquarium tanks for two months prior to the setup of the experiment. The animals were provided with a clean environment in a properly functioning aquarium under controlled condition of alkalinity, pH (6.8-7.5), temperature (26-28.5 °C), light condition 12:12 (light: dark) hardness (75–200 mg/L CaCO3), ammonia, dissolve oxygen (7.8 mg/L at 28.0 °C), salinity (0.25–0.75 ppt) and conductivity was monitored on regular basis to ensure good water quality for housing zebrafish [38]. Animals were fed 1-2 times daily of fish food. All fishes were naive, and were allowed ten days to adapt the laboratory environment before experimentation.

Collection of plant material and preparation of standardized extract

Whole plants of *Allium stracheyi* (Baker) were collected from high altitude (Munshyari Tehsil) of district Pithoragarh, Uttarakhand and were identified from the Department of Botany, LSM Govt. Post Graduate College, Pithoragarh. Air dried whole plant of *Allium stracheyi* were used for methanolic extraction by percolation. The residue obtained after removing solvent was dried in vaccum and macerated with acetone to give free flowing powder. The standard drug used in the study, benzodiazepine known anxiolytic (diazepam) was purchased from Ranbaxy, India.

Drug Adminitration

The drug was prepared immediately before use and administered through dissolving in water. Diazepam was used at the doses of 0.5, 1.0, 2.0 and 4.0 mg/litre and methanolic extract of *Allium stracheyi* as test drug was used at the dose of 50, 100, 200 and 400 mg/liter. Individual zebrafish was transferred from their home tank to a 250 ml beaker filled with 200 ml distilled water (control) or 200 ml distilled water treated with drug.

Experimental Procedure

Behavioural test were conducted between 10:00 AM to 3:00 PM. Before experimentation, zebrafish were transferred in their home cages from the animal unit to the experimental room one hour before each test session. After the habituation period in the laboratory the zebrafish were subjected to the test. In each study the zebrafish were randomly selected (n= 8) and divided into groups; Group-I placebo control and group-II standard drug (diazepam) treated and group-III test drug *(Allium stracheyi)*. Each subject is randomly assigned to a treatment group. At one time one animal from particular group immersed in a solution containing the drug/vehicle for 30 min prior to behavioural observation. Experiments were conducted 30 minutes after vehicle/drug administration to the respective group. All the apparatus were cleaned thoroughly before and after each trial to remove any trace of odour. The experiments were done in a sound attenuated room and each six minute test sessions were recorded via an overhead video camera which is used to analyze behaviour later. After six minutes the zebrafish was removed from the test tank. A number of tests session were conducted to observe the behaviour of zebrafish. All behavioural recordings were carried out with an observer not aware of the treatment and behavioural endpoints of the zebrafish. The following tests (aquatic light and dark transition and open field test) were conducted to study the effect of *Allium stracheyi* on behaviour of zebrafish.

Aquatic light/dark transition test (Scototaxis)

Scototaxis is very similar to murine light/dark box [43], which exploits the tendency of zebrafish to explore a novel environment when confronted with the aversive properties of a brightly lit area (scotophilia, scototaxis) [43]. Analogous to rodent model, zebrafish exhibit a natural preference for the dark side when given a free choice between a dark and a light chamber [44, 45, 46]. This test has been used to investigate the anxiolytic or anxiogenic properties of a variety of drugs

[47]. Studies show that anxiolytic drugs increase the exploratory behaviour and time spent in white compartment while anxiogenic drugs cause the opposite effect [47, 48, 49].

Open field test

Open field exploration task is one of the popular tests of anxiety in rats [50]. This test is readily adaptable to zebrafish and therefore is widely used [40]. In rodents, a suppression of exploratory behaviour includes freezing, thigmotaxis and a reduction in locomotor activity are the behavioural measures of anxiety but in fish freezing and thigmotaxis have been considered as indicators of anxiety [28, 51]. In open field exploration task, zebrafish initially exhibit fear [28, 52] including thigmotaxis/centrophobic behaviour as observed in rodents.

RESULTS AND DISCUSSION

Behavioural studies on aquatic animal are gradually increasing exponentially. Several assays are being used specifically to test anxiety in the zebrafish. One stimulus that causes anxiety in zebrafish is novelty indicated by the presence of the fish at the bottom periphery (thigmotaxis) of the tank. Another index of anxiety in zebrafish is the preference for dark over light environments, or scototaxis. Anxious fish displays a preference for dark surroundings and freeze when forced into light surroundings. Additionally, bottom-dwelling, leaping, hyperactivity and erratic movement have also indicators of anxiety in the 'open tank' paradigm.

The effect of *Allium stracheyi* was studied at the dose of 50, 100, 200 and 400 mg/litre and compared with control and standard drug diazepam (0.5, 1.0, 2.0, 4.0 mg/l) in open field and light and dark transition test. At all doses *Allium stracheyi* did not produce significant effects in all the behavioural paradigms of open field and light and dark transition test (Table-1 & 2). *Allium stracheyi* produced enhanced time spent by each subject in the center of the tank but which is mild and not significant while time spent by the fish in periphery was significant compared with control and diazepam. Time spent on freezing in the center, number of transition between zones and time spent on freezing in periphery were also not affected makedly compared to control. In the light and dark transition test, time spent by each subject in the white half of the tank was not significant enhanced at all doses whereas mild alterations were observed in the number of enteries and time spent in the black half of the tank and number of enteries to the white half of the tank comparerd to control animals.

Table-1: Light and Dark Transition test:

Drug/Dose n=8	Time spent in the black half of the apparatus (Seconds)	Number of enteries to the black half of the apparatus	Time spent in the white half of the apparatus (seconds)	Number of enteries to the blackwhite half of the apparatus
Control	241.375±5.560	12.125±3.173	101.875±3.739	4.5±1.467
Diazepam 0.5 mg/L	228.375±3.956	12.75±1.864	124.25±2.288	9.625±1.165
Diazepam 1.0 mg/L	181.125±7.383	9.375±1.463	143±3.932	8.125±1.359
Diazepam 2.0 mg/L	155.5±2.341	11.375±1.874	199.875±3.993	14.5±2.027
Diazepam 4.0 mg/L	179.375±3.538	7.25±1.231	180.625±3.538	9.5±1.928
Allium stracheyi 50 mg/L	218.875±4.955	13.5±1.928	92±5.508	6.75±1.149
Allium stracheyi 100 mg/L	201.125±5.354	11.375±2.380	110.5±5.836	8.625±0.983
Allium stracheyi 200 mg/L	209.25±8.611	12.375±1.246	102.25±5.040	12.625±1.463
Allium stracheyi 400 mg/L	198.375±8.567	13.125±1.847	139.25±3.448	6.125±1.359

Values represent the group mean± SEM, (n=8), P<0.05 vs. control

Table-2: Open Field Test:

Drug/Dose n=8	Time spent in the centre of the tank (seconds)	Time spent in the peripheri of the tank (seconds)	Number of transition between zones	Time spent on freezing in the center (seconds)	Time spent on freezing in periphery (seconds)
Control	66.25±4.049	242.125±5.725	13.75±1.756	16.375±1.394	33.75±1.149
Diazepam 0.5 mg/L	245.25±7.800	76.125±2.596	2±0.766	11.75±0.733	22.625±1.766
Diazepam 1.0 mg/L	309.125±4.184	28.625±4.265	6.875±0.820	6.5±0.766	13.375±1.528
Diazepam 2.0 mg/L	282±4.847	39.875±4.876	4.875±0.690	11.875±1.122	24.75±2.245
Diazepam 4.0 mg/L	246.125±5.427	54.625±1.975	1.375±0.428	29±3.221	27.875±1.429
Allium stracheyi 50 mg/L	97.875±4.795	219±3.455	12.375±1.528	15.75±1.231	27.125±2.275
Allium stracheyi 100 mg/L	87.875±5.092	237.875±4.434	11.25±1.756	13.125±1.950	22.5±1.399
Allium stracheyi 200 mg/L	98.875±2.778	217±4.335	9.625±1.651	12.5±1.824	29.125±1.285
Allium stracheyi 400 mg/L	92.125±6.311	218.75±5.286	12.75±1.864	14.375±1.766	33.625±2.119

Values represent the group mean± SEM, (n=8), P<0.05 vs. control

Anxiety has been postulated to be involved in the etiopathogenesis of psychosomatic disorders including psychiatric disorders [18]. The failure of successful adaptation during stressful situations results in anxiety disorders [53]. Prolonged stressful conditions have been associated with dysfunction of several neurotransmitters [54] resulting in behavioral changes as well as a cascade of hormonal release from the hypothalamus–pituitary–adrenal (HPA) axis leading to disorders like anxiety and depression [55, 56]. No biological cause has been identified for anxiety disorders [57]. Heisler et al (1998) [58] suggested 5HT subtype, $5HT_{1A}$ has been the main serotonin receptor implicated in fear and anxiety and $5HT_{1A}$ receptor partial or total agonist showed anxiolytic properties. According to McEwen (2000) [59] HPA axis dysregulation caused by stress results in excess production of noradrenalin and corticosterone, sensitizes peripheral inflammatory response [60], and increases anxiety [61].

Since, anxiety is associated with behavioral responses that are replicable in animal models and altered by anxiolytic drugs. In anxiety model of fish, certain predation threat (predator odors, alarm pheromones or fleeing conspecifics) are typically used for anxiogenic effects [26]. Several assays were used to test antianxiety activity of *Allium stracheyi* in the present study and also used by others [40, 45]. In light and dark transition test; when anxious, fish displays a preference for dark surroundings and they freeze when forced into light surroundings [66] which was observed in the study and these anxiogenic behaviour were mildly reverted by *Allium stracheyi* compared to control while benzodiazepines (diazepam) know anxiolytic significantly enhanced the the time spent in light area of the tank. In open field test [50], in which suppression of exploratory behaviour including freezing and thigmotaxis have been considered as indicator of anxiety (28, 51). *Allium stracheyi* at all doses produce mild decrease in the time spent in the periphery (thigmotaxis) of the tank, number of transition between zones, freezing in the center and periphery of the tank compared to control and diazepam. Additionally, bottom-dwelling (or diving), leaping, hyperactivity, and erratic movement have also been suggested as species-specific indicators of anxiety in the 'open tank' paradigm [27, 67]. The novel tank test is an alternative to open field test and commonly used to assess zebrafih behaviour and anxiety paradigms [39, 40, 41, 42]. This test exploits the natural tendency of zebrafish to seek refuge when exposed to a novel environment [27]. The mild anxiolytic effects of *Allium stracheyi* may have some other type of activity. Mechanism of action of anxiolytic plants may have interaction with some of the natural endogenous mediators in the body as reported by several scientific communities [62, 63]. It is evident that there could be a linkage in the interaction of serotonergic pathways and plant extract [64, 65]. 5HT subtype, $5HT_{1A}$ has been considered the main serotonin receptor implicated in fear and anxiety and $5HT1_A$ receptor partial or total agonist showed anxiolytic properties [58]. Breier and Paul [14], indicated that benzodiazepines/γ-aminobutyric acid (BZ/GABA)

receptor complex is involved in the pathogenesis of anxiety and benzodiazepines produce their effects by facilitating GABA neurotransmission [13].

A recent study has shown that both basic and complex brain phenomena as well as endocrine mechanisms, share a substantial homology between zebrafish and mammals such as mice and men [68]. Zebrafish nervous system and brain aminergic systems share many structural properties with the mammalian systems (including humans) [69]. Since zebrafish possess all the classical vertebrate neurotransmitters, and their neuroendocrine system yields robust cortisol responses to stress, thus zebrafish model enable greater insight into neural mechanisms associated with anxiety-related disorders. Therefore zebrafish were used in the present study which serves as inexpensive and high-throughput models for the development of anxiolytic drugs. Benzodiazapine GABA-A receptors have been found in a wide variety of species including bony fishes [70] and have similar binding characteristics as those in rodents and humans [71-74]. Zebrafish have similar types of muscrinic and acetylcholine neurotransmitter receptors to the mammals. Studies have examined anxiety in zebrafish using anxiolytic agents such as nicotine [27], α-fluromethylhistidine 75, ethanol [76] and diazepam [28]. Drug-evoked anxiety has also been reported in zebrafish using the benzodiazepine inverse agonist FG-7142 or following abrupt cessation of chronic cocaine administration [28].

Zebrafish brain aminergic system has many structural properties common to the mammalian systems. The noradrenergic, serotonergic, aminergic and histaminergic systems of zebrafish are highly similar to the mammalian system [77-79]. The basic and complex brain phenomena as well as endocrine mechanisms of zebrafish and mammals are substantially homologous [68]. Just like humans, zebrafish employs cortisol as the primary stress response hormone unlike corticosterone by rodents [80]. The hypothalamus pituitary inter renal (HPI) axis of zebrafish is homologous to the hypothalamus pituitary adrenal (HPA) axis of humans. Corisol is the primary stress hormone in both species [81]. Zebrafish model enables greater insight into neural mechanism associated with anxiety related disorders since it possess all the classical vertebrates' neurotransmitters and its neuroendocrine system yields robust cortisol responses to stress. There are several characteristics which make zebrafish an important test subject which could prove useful in a greater understanding of neuropharmacological mechanisms in mammals and facilitate behaviour based drug discovery [24]. Zebrafish have robust physiological responses and quantifiable behavioural and neuropathological phenotypes analogous to humans [82]. It also offers an alternative and efficient mode of drug delivery via the gills [27, 51, 10]. Beneficial property like low maintenance cost and rapid life cycle make easy to maintain a large number of zebrafish in a relatively small area which is important for large scale behavioural studies and psychopharmacological screening of medicinal plants for drug development [24, 40].

Overall observation indicates when a zebrafish was presented to an unfamiliar environment it showed robust anxiety-like behavioural responses, which were measured in open field test and light/dark transition. Methanolic extract of *Allium stracheyi* reverted anxiety-like behavioural responses in zebrafish but not significantly compared to control and diazepam. This indicates that crude extract of *Allium stracheyi* imparted mild anxiolytic property. Further studies are needed to isolate and characterize the active component of *Allium stracheyi* to evaluate their antianxiety effect in various behavioural test models.

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